

AMENDMENTS TO THE SPECIFICATION

Please insert the attached written Sequence Listing after the specification, but before the claims.

Please delete the paragraph starting on page 20, line 16 and ending on page 20, line 21, and replace it with the following paragraph:

In FIGURE 4 and its adjunct Table the chromatographic profile of the tryptic digestion of the diabody, and the summary of the assignation of the tryptic peptides of the diabody, can be seen. In the ESI-MS spectra no signals indicating incorrectly linked cysteines were detected, evident from the summaries of the fractions 8 and 12 of the Table adjunct to FIGURE 4, that contains the peptides (²⁰Phe-Arg³¹)-S-S-(⁸⁷Ser-Arg⁹⁷) (SEQ ID NO: 22) and (¹⁴³Val-Lys¹⁴⁸)-S-S-(¹⁸⁶Ile-Lys²²⁸) (SEQ ID NO: 23) linked by disulphide bonds (-S-S-) between cysteines 22 and 95, and 147 and 212, respectively.

Please delete the paragraph starting on page 10, line 38 and ending on page 11, line 6, and replace it with the following paragraph:

Total RNA from 10⁶ cells of the mouse hybridoma CB/ior-CEA.1 (Tormo B. et al. APMIS. 97: 1073-1080, 1989) was extracted with the TriPureTM reagent TRIPURETM isolation reagent (Boehringer-Mannheim). The complementary DNA (cDNA) was synthesized using the First-Strand cDNA Synthesis for RT-PCR Kit (Boehringer-Mannheim), using oligo dT as primer. The polymerase chain reaction technique (PCR) for the specific amplification of the heavy and light chain variable domain genes was used. The employed synthetic primers were designed on the basis of the consensus sequences for mouse IgG and kappa chains, reported by Kabat E. et al. (US Department of Health and Human Services, NIH, 1991) and experiments developed previously in this

laboratory (Coloma, MJ et al. Biotechniques 11: 152-156, 1991). The sequences of the oligonucleotides used in the PCR appear in Table I.

Please delete the paragraph starting on page 25, line 17 and ending on page 25, line 29, and replace it with the following paragraph:

The B16-CEA13 cells used in these experiments were obtained through the transfection of a gene that encodes for the extracellular domains of human CEA, cloned in the pDisplay™ vector (Cat. No. V660-20, Invitrogen). The gene was obtained by PCR from RNA extracted from CRL-1682 cells, with oligonucleotides designed after the published sequence of human CEA. The recombinant plasmid pDisplay-CEA was purified and transfected into C57Bl/6 mouse B16-F10 melanoma cells (ATCC CRL-6475) using Lipofectamine LIPOFECTAMINE PLUS™ transfection reagent (Gibco-BRL) and 5 µg of DNA per transfection. The selection of stable transfectants was done with 4.0 mg/mL of geneticin sulphate (G418; Gibco-BRL) for 14 days, after which the surviving cultured cells were cloned by limiting dilution and those clones that expressed human CEA in their surface were identified through indirect immunofluorescence, using Mab CB/ior-CEA.1 as first antibody, and anti mouse IgG antibodies conjugated with FITC (Sigma) for development. It was found that 73% of the clones presented more than 80% of the cells with specific membrane fluorescence indicating that the human CEA was exposed correctly folded and glycosylated in their surface.

Please delete the paragraph starting on page 3, line 20 and ending on page 3, line 24, and replace it with the following paragraph:

The scFv monovalent and diabody fragments reported in this invention have important differences in ~~aminoacids~~ amino acids in the heavy chain (VH, and light chain (VL) variable domains, with respect to other scFv previously

developed from the same Mab, and surpass it in affinity for CEA, in performance for the recognition of cells and tissues, and in efficacy for the localization of tumors that produce human CEA growing *in vivo* in mice.

Please delete the paragraph starting on page 3, line 20 and ending on page 3, line 24, and replace it with the following paragraph:

The recombinant scFv monovalent and diabody fragments reported in this invention were developed using PCR, and cloning and expression techniques in recombinant microorganisms, starting from the RNA extracted from the CB/ior-CEA.1 hybridoma. Sets of oligonucleotides different from those used to obtain a previously reported scFv (Ayala et al. Biotechniques 13: 790-799, 1992), were employed for the amplification and isolation of the base sequences encoding the Mab VH and VL domains. In the invention it is shown that the new monovalent and diabody scFv have important differences in the ~~amino acid~~ amino acid sequences of the VH and VL domains, with respect to a scFv previously obtained, and that these take the form of 16 ~~amino acids~~ amino acids in the frameworks 1 (FR1) and 3 (FR3) and in the complementary determinant region 2 (CDR2) of the VH domain, different with respect to the scFv previously obtained, and 3 ~~amino acids~~ amino acids between the FR1 and FR3 of the VL domains, different with respect to the scFv previously obtained. This indicates that these domains have a different clonal origin with respect to those reported in Ayala et al. Biotechniques 13: 790-799, 1992. In the case of the diabody, this one also differs from the scFv previously obtained in the size and ~~amino acidie~~ amino acidic composition of the union segment (linker) that is employed in the fabrication of the scFv-type molecule.

Please delete the paragraph starting on page 3, line 39 and ending on page 4, line 7, and replace it with the following paragraph:

The changes reflect surprisingly in the biochemical and biological properties of the new fragments, and provide them with a behavior very similar to the Mab CB/ior-CEA.1, and very much superior to that of the previously reported scFv. The new monovalent scFv fragment, that has a linker identical to the previously reported scFv (Ayala et al. Biotechniques 13: 790-799, 1992), but the aforementioned ~~amino acid~~ amino acid changes in the variable domains, has an affinity constant for human CEA very much higher than the previously reported scFv. Also, the diabody surpasses both monovalent scFv forms in its affinity constant for human CEA. The two new scFv monovalent and diabody fragments conserve the properties of specificity of the original Mab with respect to CEA recognition, identification of tumor cells and tissues, absence of cross reactivity with NCA, and capacity to accumulate selectively in a tumor that produces human CEA transplanted in mice, all with a very much superior performance than that of the previously obtained scFv.

Please delete the paragraph starting on page 4, line 13 and ending on page 4, line 19, and replace it with the following paragraph:

In the present invention it is shown how it is possible to amplify by PCR the VH and VL domains of the Mab CB/ior-CEA.1 using synthetic oligonucleotides that hybridize in the base sequences that encode for the signal peptides and constant domains CH1 and Ck. It is also shown the possibility of assembly of the amplified VH and VL domains, in this order, using PCR, and obtaining different forms of scFv fragments manipulating the size of the linker that connects the domains. Using 14 ~~amino acids~~ amino acids a monovalent scFv form is originated, and reducing this number to five, a diabody scFv type form is produced.

Please delete the paragraph starting on page 6, line 8 and ending on page 6, line 11, and replace it with the following paragraph:

The polypeptide molecule in the form of a monovalent scFv fragment specific for human CEA exhibits an affinity constant for this antigen of $(5.0 \pm 0.4) \times 10^9 \text{ L mol}^{-1}$, and comprises the VH and VL domains, linked in this order by a 14-~~aminoacid~~ amino acid union segment (linker), with an ~~aminoacid~~ amino acid sequence as the one presented in SEQ ID No. 16.

Please delete the paragraph starting on page 6, line 12 and ending on page 6, line 16, and replace it with the following paragraph:

The polypeptide molecule in the form of a divalent scFv fragment (diabody) specific for human CEA exhibits an affinity constant for this antigen of $(2.8 \pm 0.3) \times 10^{10} \text{ L mol}^{-1}$, and comprises the pairing of two identical molecules formed each one by the VH and VL domains, linked in this order by a five-~~aminoacid~~ amino acid union segment (linker), with an ~~aminoacid~~ amino acid sequence as the one presented in SEQ ID No. 17.

Please delete the paragraph starting on page 7, line 13 and ending on page 7, line 17, and replace it with the following paragraph:

Apart from the antibody sequences, the polypeptide molecules contained in this invention can comprise other ~~aminoacids~~ amino acids that form a peptide or polypeptide, or that add to the molecule a functional characteristic different to that of binding the CEA antigen, as for example a tag for purification or identification, an enzyme or its fragments, a biological response modifier, a toxin or drug, and successively.

Please delete the paragraph starting on page 8, line 35 and ending on page 8, line 39, and replace it with the following paragraph:

The scFv monovalent and diabody fragments and their equivalent variant forms in agreement with the present invention can be fabricated through the expression of the encoding nuclei acid. The nucleic acid that encodes for any of these polypeptide molecules described before is part of the present invention, as it is a method for the expression of such nucleic acid. In a different embodiment, the nucleic acid can encode for the ~~aminoacid~~ amino acid sequences shown in SEQ ID No. 16 and 17.

Please delete the paragraph starting on page 9, line 29 and ending on page 9, line 33, and replace it with the following paragraph:

In FIGURE 2 the alignment of the ~~aminoacid~~ amino acid sequences (in one-letter code) deducted from the nucleotide ones for (1) the monovalent scFv fragment (SEQ ID No. 16), and (2) the divalent fragment (diabody) (SEQ ID No. 17), are presented. The order of the domains in both constructions are VH-linker segment-VL. The ~~aminoacids~~ amino acids of the linker segments employed in each of the two molecules appear in bold characters.

Please delete the paragraph starting on page 10, line 3 and ending on page 10, line 8, and replace it with the following paragraph:

FIGURE 5 is a summary of the ~~aminoacids~~ amino acids sequence verification of the diabody (SEQ ID No. 21). The regions of the protein sequence that were verified by mass spectrometry are outlined in bold characters, and the zones of the sequence that were not recovered after tryptic digestion appear in

italics. The zones in bold coincide in total with the ~~aminoacid~~ amino acid sequence deducted from the base sequence of the diabody. The sequence of the c-myc peptide and the final 6 histidines, provided by the pJG-1m vector (FIGURE 1), are also seen in the C-terminus portion.

Please delete the paragraph starting on page 12, line 3 and ending on page 12, line 6, and replace it with the following paragraph:

The synthetic oligonucleotides were designed on the basis of the sequences of VH and VL in the plasmids pVH5 and pVL2. These included sites of restriction for the cloning in the vector pJG-1m and incorporated the linker segments of 14 and 5 ~~aminoacids~~ amino acids for the assembly of monomeric scFv and diabody (Tables II and III).

Please delete the paragraph starting on page 12, line 8 and ending on page 12, line 9, and replace it with the following paragraph:

Table II. ~~Aminoacid~~ Amino acid sequences of the union segments (linkers) used for the construction of the scFv and diabody fragments.

Please delete the paragraph starting on page 12, line 13 and ending on page 12, line 27, and replace it with the following paragraph:

Table III Synthetic oligonucleotides used in the PCR for the assembly of scFv and diabody.

Oligo 5. ApaL1- FR1 VH (SEQ ID No. 7)

5'...TCTCACAGTGCACAGGAAGTGAAGCTGGTGGAGTCTGGG...3'

Oligo 6. Linker of 14 ~~aminoacids~~ amino acids /FR4 VH (SEQ ID No. 8)

5'...GTCGACTTTGGATTCCGAGCCTGATCCTGAGGATTTACCCTCTG
AGGAGACTGTGAGAGTGGT...3'

Oligo 7. Linker of 14 ~~aminoacids~~ amino acids /FR1 VL (SEQ ID No. 9)

5'...GAGGGTAAATCCTCAGGATCAGGCTCCGAATCCAAAGTCGACG
ACATTGTGATGACCCAGTC...3'

Oligo 8. Not I- FR4 VL (SEQ ID No. 10)

5'... AAGGAAAAAAGCGGCCGCTTTCAGCTCCAGCTTGGTT...3'

Oligo 9. Linker of 5 ~~aminoacids~~ amino acids /FR4 VH (SEQ ID No. 11)

5'... AGAGCCGCCGCCACCTGAGGAGACTGTGAGAGTGGT...3'

Oligo 10. Linker of 5 ~~aminoacids~~ amino acids /FR1 VL (SEQ ID No. 12)

5'... GGTGGCGGCGGCTCTGACATTGTGATGACCCAGTCT...3'

Please delete the paragraph starting on page 13, line 40 and ending on page 14, line 4, and
replace it with the following paragraph:

The ~~aminoacid~~ amino acid sequences derived from the base sequences obtained for the monovalent scFv (clone pJG1m-25) and the diabody (clone pJG1m-18) appear in FIGURE 2 (SEQ ID No. 16 and SEQ ID No. 17). With respect to a scFv developed previously (Ayala M et al. Biotechniques 13: 790-799, 1992), the VH and VL sequences now obtained for the new monovalent scFv and diabody exhibit 16 different ~~aminoacids~~ amino acids within the VH FR1, CDR2 and FR3 domains, and 3 different ~~aminoacids~~ amino acids within the VL FR1 and FR3 domains.

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Please delete the paragraph starting on page 20, line 22 and ending on page 20, line 27, and replace it with the following paragraph:

From the peptides analyzed by ESI-MS, 92% of the diabody sequence could be obtained in a single proteolytic digestion (FIGURE 5). In this sequence there is a full coincidence with the ~~amino acid~~ amino acid sequences deduced from the base sequence of the VH and VL domains (SEQ ID No. 16 and 17), amplified by PCR starting from the total RNA of the hybridoma that produces the Mab CB/ior-CEA.1, of the 5-~~amino acid~~ amino acid linker segment (SEQ ID No. 10), and in the C-terminal portion, of the sequence of the c-myc peptide and 6 final histidines, provided by vector pJG-1m (FIGURE 2).